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Total synthesis of biologically active 20S-hydroxyvitamin D3

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Abstract

A total synthetic strategy of 20S-hydroxyvitamin D3 [20S-(OH)D3] involving modified synthesis of key intermediates **7** and **12**, Grignard reaction to stereoselectively generate 20S-OH and Wittig-Horner coupling to establish D3 framework, was completed in 16 steps with an overall yield of 0.4 %. The synthetic 20S-(OH)D3 activated vitamin D receptor (VDR) and initiated the expression of downstream genes. In addition, 20S-(OH)D3 showed similar inhibitory potency as calcitriol [1,25(OH)₂D3] on proliferation of melanoma cells.

Keywords

20S-hydroxyvitamin D3; total chemical synthesis; PCR; reporter assay; thymidine incorporation; VDR activation

1. Introduction

25-hydroxylase (CYP2R1 or CYP27A1) in the liver and 1 α -hydroxylase (CYP27B1) in the kidney are the enzymes responsible for sequential metabolism of vitamin D3 to produce bioactive 1 α ,25-dihydroxyvitamin D3 [1,25(OH)₂D3, calcitriol] [1, 2]. 1,25(OH)₂D3 activates nuclear vitamin D receptor (VDR), which is found in almost all tissues of the body, to trigger numerous cellular effects including but not limited to stimulation of cell differentiation and/or apoptosis, inhibition of cell proliferation, regulation of secretory and immune factors of many cells, and cell protective functions, in a context dependent fashion

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:

[3–7]. Therefore, active forms of vitamin D can be used in therapy of cancer, hyperproliferative, autoimmune and metabolic disorders [8–10]. However, these applications are limited by hypercalcemic (toxic) effect of calcitriol at pharmacological concentrations [11]. This side effect has led to the development of more than 3,000 synthetic vitamin D3 analogs showing low calcemic activity [12]. Some of these analogs such as paricalcitol, oxacalcitriol, falecalcitriol, tacalcitol and doxercalciferol were used to treat secondary hyperparathyroidism and psoriasis [8, 13]. Besides psoriasis, calcipotriol is also a promising agent for the treatment of pancreatic cancer and is currently undergoing human clinical trial [14] (Figure 1).

Mammalian cytochrome P450 side-chain cleavage enzyme (P450_{scc} or CYP11A1) not only cleaves the side chain of cholesterol to produce pregnenolone (precursor of all steroids) [15, 16] but also hydroxylates vitamin D3 in a sequential fashion [16–20] starting from C20 to form 20S-hydroxyvitamin D3 [20S-(OH)D3] (**1**, Figure 1), which is subsequently converted to di- and trihydroxy metabolites [17, 19–22]. Functional studies showed that 20S-(OH)D3 not only stimulated keratinocyte differentiation program but also inhibited NF-κB activity in human keratinocytes [23]. In addition, it has shown anti-inflammatory activities, strong anti-proliferative effects, anti-leukemia and tumorostatic effects [23–27], protective effects against ultra-violet B (UVB) induced damage [28], as well as antifibrotic activity *in vivo* [29]. These activities are mediated either through activation of the VDR [23, 30] or inhibition of RORα and RORγ transcriptional activities [31]. More importantly, while having comparable anti-proliferative potency with 1,25(OH)₂D3 which has strong hypercalcemic toxicity at a concentration of 1 μg/kg, 20S-(OH)D3 is not hypercalcemic at concentrations as high as 60 μg/kg [24, 26, 28, 32].

Unfortunately, in-depth evaluation of the biological activity for 20S-(OH)D3 or its analogs was hampered by lack of an efficient synthetic method of its production [33] without using costly enzymatic approaches [20, 21, 34]. 20S-(OH)D3 was firstly chemically synthesized from pregnenolone acetate via Grignard reaction and low yielding UVB irradiation to afford low milligram scale of 20S-(OH)D3 stereo-specifically. However, this method also generated structurally similar and physicochemically active by-products including previtamin D3, lumisterol and tachysterol which presented a significant challenge in the purification of 20S-(OH)D3 even through preparative HPLC [33]. Due to these disadvantages, scope of synthesis through UVB irradiation of 5,7-dienal precursor, 20S-(OH)-7-dehydrocholesterol was confined to very few derivatives, if not to almost exclusive production of 20S-(OH)D3. Therefore, an approach that could easily generate large quantity of 20S-(OH)D3 with high quality (> 98% purity) would facilitate further modifications on the side chain to determine the structure-activity relationships of this promising 20S-hydroxyl scaffold. Herein, we report an efficient total synthetic route for stereospecific 20S-(OH)D3.

2. Experimental

2.1. General methods

Tetrahydrofuran was distilled from sodium-benzophenone. All other solvents and chemical reagents were obtained from commercial sources and directly used without further

purification. Ergocalciferol was purchased from Chem Impex International Inc. Glassware was oven-dried before use. All reactions were performed under an argon atmosphere. TLC was performed on silica gel 60 GF254 and monitored under UV light or visualized using phosphomolybdic acid reagent. Flash chromatography was performed on 230–400 mesh silica gel (Fisher Scientific). Preparative TLC was performed on Analtech TLC Uniplates (250 μ m). Melting points were recorded on a MPA100 Automated Melting Point Apparatus. NMR spectra were obtained on a Bruker Ascend 400 (Billerica, MA) spectrometer or a Varian Inova-500 spectrometer (Agilent Technologies, Santa Clara, CA). Chemical shifts are given in ppm with tetramethylsilane (TMS) as an internal reference. All coupling constants (J) are given in Hertz (Hz).

2.2. Chemistry

2.2.1. (4R,7aR)-1-((2R,5R,E)-5,6-dimethylhept-3-en-2-yl)-4-((R,Z)-1-hydroxy-2-((S)-5-hydroxy-2-methylenecyclohexylidene)ethyl)-7a-methyloctahydro-1H-inden-4-ol (2)—Following a reported procedure [35], to a solution of ergocalciferol (20 g, 50.5 mmol) in ethanol (2 L) at -45°C was added dropwise solution of potassium permanganate (9.0 g, 58.7 mmol) in water (300 mL), the mixture was stirred for half an hour and further 1 hour at -15°C and then warmed to room temperature. After 3 hours, the precipitate was filtered off and the solution was evaporated to give yellowish crude oil. The crude oil was purified on silica gel column chromatography eluting with hexane/ethyl acetate (3:1) to give pure triol compound **2** as white solid (10.5 g, 65%) and unreacted starting material (5.1 g). Mp: $170\text{--}172^{\circ}\text{C}$ (MeOH). ^1H NMR (400 MHz, CD_3OD): δ 5.52 (dd, $J = 9.9, 1.7$ Hz, 1H, 6-H), 5.30 – 5.09 (m, 2H, 22/23-H), 5.02 – 4.98 (m, 2H, 7/19-H), 4.90 (d, $J = 9.9$ Hz, 1H, 19-H), 3.65 (m, 1H, 3-H), 2.55 (ddd, $J = 12.4, 4.5, 1.9$ Hz, 1H), 2.49 – 2.38 (m, 1H), 2.17 – 1.90 (m, 5H), 1.90 – 1.68 (m, 4H), 1.62 (m, 1H), 1.56 – 1.39 (m, 4H), 1.39 – 1.03 (m, 5H), 1.00 (d, $J = 6.6$ Hz, 3H, 21-H), 0.94 (d, $J = 6.8$ Hz, 3H, 28-H), 0.89 – 0.81 (t, $J = 6.4$ Hz, 9H, 18/26/27-H). ^{13}C NMR (101 MHz, CD_3OD): δ 147.35, 140.78, 137.06, 133.12, 126.72, 111.72, 76.25 (C7), 71.42 (C8 or C3), 71.39 (C3 or C8), 61.04, 58.79, 47.60, 45.14, 44.36, 41.70, 41.53, 39.23, 37.21, 34.50, 34.38, 29.13, 22.87, 21.76, 21.29, 20.52, 20.12, 18.27, 13.59. ESI-HRMS: calculated for $\text{C}_{28}\text{H}_{46}\text{O}_3\text{Na}$ $[\text{M}+\text{Na}]^+$ 453.3345, found 453.3352.

2.2.2. (S,Z)-3-(2-hydroxyethylidene)-4-methylenecyclohexanol(3) and (4S, 7aR)-1-((2R,5R,E)-5,6 - dimethylhept-3-en-2-yl)-7a-methyloctahydro-1H-inden-4-ol (4)—To a suspension of **2** (6.0 g, 13.9 mmol) and sodium carbonate (14.6 g, 138 mmol) in dichloromethane (50 mL) was added lead tetraacetate (6.7 g, 15.3 mmol) in portions at 0°C . After 2 hours, the reaction was quenched with ethylene glycol and the mixture was vigorously stirred at 0°C for 10 minutes. Water was then added and the mixture was extracted with dichloromethane for five times, washed with brine and dried with Na_2SO_4 . The combined extracts were evaporated to give crude oil mixture, which was used for next step without further purification. To a solution of the above crude oily mixture in benzene (100 mL) at 0°C under argon with stirring, Red-Al (28.7 mmol, 8.6 mL) was added dropwise and the mixture was stirred for 2 hours. On completion reaction was quenched with water. The mixture was filtered off and extracted with ethyl acetate, washed with brine and dried with anhydrous Na_2SO_4 . Organic phase was evaporated to generate

crude oil mixture which was purified by flash chromatography on silica. Elution with hexane/ethyl acetate (10:1) gave pure alcohol compound **4** as transparent oil (3.3 g, 86% for two steps), further elution with hexane/ethyl acetate (1:2) gave pure allylic alcohol **3** as colorless oil (0.91 g, 43% for two steps). compound **3**: ^1H NMR (400 MHz, CDCl_3): δ 5.45 (t, $J = 6.6$ Hz, 1H, 6-H), 4.97 (dd, $J = 2.4, 1.3$ Hz, 1H, 19-H), 4.59 (d, $J = 2.3$ Hz, 1H, 19-H), 4.25 (ddd, $J = 12.7, 7.0, 1.2$ Hz, 1H, 7-H), 4.14 (ddd, $J = 12.6, 6.2, 1.3$ Hz, 1H, 7-H), 3.94 (m, 1H, 3-H), 2.56 – 2.33 (m, 2H), 2.24 (dd, $J = 13.2, 7.1$ Hz, 1H), 2.19 – 2.06 (m, 1H), 1.87 (m, 1H), 1.75 – 1.60 (m, 1H). ^{13}C NMR (101 MHz, CDCl_3): δ 144.51 (C10), 139.11 (C5), 125.95 (C6), 112.23 (C19), 68.64 (C3), 59.34 (C7), 44.82, 34.62, 31.45. ESI-HRMS: calculated for C_9H_{11} , $[\text{M}-2\text{H}_2\text{O}+\text{H}]^+$ 119.0861, found 119.0861. compound **4**: ^1H NMR (400 MHz, CDCl_3): δ 5.25 – 5.02 (m, 2H, C22/23-H), 4.02 (d, $J = 2.8$ Hz, 1H, C8-H), 2.04 – 1.88 (m, 2H), 1.86 – 1.73 (m, 3H), 1.73 – 1.60 (m, 1H), 1.56 – 1.33 (m, 5H), 1.33 – 1.02 (m, 5H), 0.96 (d, $J = 6.6$ Hz, 3H, 22-H), 0.92 (s, 3H, C18-H), 0.89 (d, $J = 11.2$ Hz, 3H, 23-H), 0.80 (t, $J = 6.6$ Hz, 6H, C26/27-H). ^{13}C NMR (101 MHz, CDCl_3): δ 135.59, 131.70, 69.14 (C8), 56.48, 52.72, 42.78, 41.65, 40.32, 39.75, 33.53, 33.00, 27.57, 22.46, 20.71, 19.88, 19.55, 17.57, 17.40, 13.61. ESI-HRMS: calculated for $\text{C}_{19}\text{H}_{33}$ $[\text{M}-\text{H}_2\text{O}+\text{H}]^+$ 261.2582, found 261.2582.

2.2.3. (S,Z)-tert-butyl(2-(5-((tert-butyldimethylsilyl)oxy)-2-

methylenecyclohexylidene)ethoxy)dimethylsilane (5)—Imidazole (1.4 g, 21.2 mmol) and *tert*-butyldimethylsilyl chloride (4.8 g, 31.6 mmol) was sequentially added to a solution of compound **3** (0.82 g, 5.3 mmol) in dichloromethane (50 mL) in a round bottom flask with stirring. The resulting suspension was stirred for 2 hours. Water was then added and the mixture was extracted with dichloromethane, washed with brine and dried with anhydrous Na_2SO_4 . Evaporation under vacuum gave the oily residue which was subjected to flash chromatography on silica. Elution with hexane/ethyl acetate (80:1) gave pure compound **5** as colorless oil (1.8 g, 88%). ^1H NMR (400 MHz, CDCl_3): δ 5.38 (ddt, $J = 6.8, 5.3, 1.3$ Hz, 1H, 6-H), 4.95 (p, $J = 1.1$ Hz, 1H, 19-H), 4.57 (dd, $J = 2.4, 1.3$ Hz, 1H, 19-H), 4.31 (ddt, $J = 13.0, 7.2, 0.9$ Hz, 1H, 7-H), 4.21 (ddd, $J = 12.9, 5.3, 2.1$ Hz, 1H, 7-H), 3.82 (tt, $J = 8.9, 3.9$ Hz, 1H, 3-H), 2.47 – 2.31 (m, 2H), 2.22 – 2.13 (m, 1H), 2.06 (m, 1H), 1.93 – 1.80 (m, 1H), 1.56 (m, 1H), 0.89 (d, $J = 4.7$ Hz, 18H, TBS), 0.08 – 0.03 (s, 12H, TBS). ^{13}C NMR (101 MHz, CDCl_3): δ 144.89 (C10), 138.24 (C5), 126.29 (C6), 111.95 (C19), 70.11 (C3), 60.59 (C7), 45.93, 36.03, 32.15, 26.01 (TBS), 25.86 (TBS), 18.43, 18.18, –4.65 (TBS), –4.69 (TBS), –4.99 (TBS), –5.05 (TBS). ESI-HRMS: calculated for $\text{C}_{21}\text{H}_{42}\text{O}_2\text{Si}_2\text{Na}$ $[\text{M}+\text{Na}]^+$ 405.2621, found 405.2629.

2.2.4. (S,Z)-2-(5-((tert-butyldimethylsilyl)oxy)-2-

methylenecyclohexylidene)ethanol (6)—To a solution of compound **5** (1.7 g, 4.4 mmol) in anhydrous THF (20 mL) in a round bottom flask at -5°C under nitrogen, *tetra-n*-butylammonium fluoride (1.0 M in THF, 4.8 mmol) was added dropwise with stirring. The resulting mixture was stirred for 3 hours, water was then added and the brown mixture was extracted with dichloromethane, washed with brine and dried with anhydrous Na_2SO_4 . The combined extracts were evaporated under vacuum to give the oily residue which was purified with flash chromatography on silica. Pure allylic alcohol **6** was eluted out with hexane/ethyl acetate (50:1) as colorless oil (0.93 g, 78%). ^1H NMR (400 MHz, CDCl_3): δ

5.44 (ddt, $J = 7.3, 5.9, 1.3$ Hz, 1H, 6-H), 4.96 (d, $J = 1.2$ Hz, 1H, 19-H), 4.62 (t, $J = 2.4$ Hz, 1H, 19-H), 4.43 – 4.01 (m, 2H, 7-H), 3.84 (m, 1H, 3-H), 2.48 – 2.33 (m, 2H), 2.26 – 2.14 (m, 1H), 2.13 – 2.01 (m, 1H), 1.87 (m, 1H), 1.58 (m, 1H), 0.88 (s, 9H, TBS), 0.06 (d, $J = 2.9$ Hz, 6H, TBS). ^{13}C NMR (101 MHz, CDCl_3): δ 144.83, 140.52, 124.88, 111.91, 70.11 (C3), 59.82 (C7), 46.02, 35.98, 32.26, 25.82 (TBS), 18.15, –4.67 (TBS), –4.71 (TBS). ESI-HRMS: calculated for $\text{C}^{15}\text{H}_{28}\text{O}_2\text{SiNa}$ $[\text{M}+\text{Na}]^+$ 291.1756, found 291.1755.

2.2.5. (S,Z)-(2-(5-((tert-butyldimethylsilyl)oxy)-2-

methylenecyclohexylidene)ethyl)diphenylphosphine oxide (7)—To a solution of the allylic alcohol **6** (0.81 g, 3.0 mmol) in anhydrous THF (20 mL) was added *n*-BuLi (2.5 M in hexanes, 30.0 mmol) under argon at 0 °C with stirring. After 5 minutes, a solution of *p*-tosyl chloride (598 mg, 3.15 mmol) dissolved in anhydrous THF (10 mL) was added to the allylic alcohol-BuLi solution. On the other side, to a solution of diphenylphosphine (1.02 mL, 6.0 mmol) in anhydrous THF (5 mL) under argon at 0 °C with stirring *n*-BuLi (2.5 M in hexanes, 2.2 mL, 5.7 mmol) was added dropwise to provide a red solution. After 10 minutes 4.1 mL of the red solution was then drawn to add to the solution of tosylate. The mixture was stirred for 30 minutes at 0 °C and quenched with H_2O (1 mL). Evaporation of the solution under vacuum gave a phosphine residue. To the phosphine residue in dichloromethane (15 mL) at 0 °C with stirring was added 10% H_2O_2 (1.1 mL). Reaction was quenched with water, extracted with dichloromethane, washed with cold saturated sodium sulfite solution and brine and dried with Na_2SO_4 . Organic solvent was removed under vacuum to give crude residue which was subjected to flash chromatography on silica for purification. Elution with hexane/ethyl acetate (3:1) gave pure phosphine oxide compound **7** as white solid (800 mg, 59%). Mp: 94–96 °C (EtOAc). ^1H NMR (400 MHz, CDCl_3): δ 7.71 (m, 4H, Ph), 7.56 – 7.36 (m, 6H, Ph), 5.35 (q, $J = 7.2$ Hz, 1H, 6-H), 4.92 (t, $J = 1.9$ Hz, 1H, 19-H), 4.68 (dd, $J = 2.4, 1.3$ Hz, 1H, 19-H), 3.52 (m, 1H, 3-H), 3.36 (m, 1H, 7-H), 3.20 (m, 1H, 7-H), 2.44 – 2.31 (m, 1H), 2.22 (dt, $J = 13.3, 4.7$ Hz, 1H), 2.15 – 2.05 (m, 1H), 1.81 – 1.71 (m, 1H), 1.66 (m, 1H), 1.47 (m, 1H), 0.83 (s, 9H, TBS), –0.00 (d, $J = 2.0$ Hz, 6H, TBS); ^{13}C NMR (101 MHz, CDCl_3): δ 145.03, 145.01, 142.62, 142.50, 133.49, 132.99, 132.52, 132.00, 131.75, 131.73, 131.61, 131.58, 131.20, 131.11, 130.95, 130.86, 128.59, 128.48, 128.44, 128.32, 113.19, 113.11, 111.60, 111.59, 70.46 (C3), 70.43, 46.68, 46.66, 36.21, 32.48, 32.46, 31.44, 30.74, 25.82 (TBS), 18.12, –4.69 (TBS), –4.75 (TBS). ESI-HRMS: calculated for $\text{C}_{27}\text{H}_{37}\text{O}_2\text{PSiNa}$ $[\text{M}+\text{Na}]^+$ 475.2198, found 475.2203.

2.2.6. (4S,7aR)-1-((2R,5R,E)-5,6-dimethylhept-3-en-2-yl)-7a-

methyloctahydro-1H-inden-4-yl acetate (8)—Pyridine (1.3 mL, 16.1 mmol), 4-dimethylaminopyridine (352 mg, 2.88 mmol) and acetyl anhydride (0.98 mL, 104 mmol) was sequentially added to a solution of compound **4** (3.2 g, 11.5 mmol) in anhydrous dichloromethane (30 mL) in a round bottom flask under argon. The resulting mixture was stirred for 3 hours. Water was then added and the mixture was extracted with dichloromethane, washed with saturated NaHCO_3 , 0.1 M HCl and brine and then dried with Na_2SO_4 . Evaporation under vacuum gave the oily residue which was purified with flash chromatography on silica. Elution with hexane/ethyl acetate (30:1) gave pure compound **8** as colorless oil (3.4 g, 92%). ^1H NMR (400 MHz, CDCl_3): δ 5.31 – 5.00 (m, 3H, 8/22/23-H), 2.12 – 1.92 (m, 5H), 1.92 – 1.53 (m, 4H), 1.53 – 1.03 (m, 10H), 0.99 (d, $J = 2.4$ Hz, 3H, 22-

H), 0.94 – 0.85 (m, 6H), 0.82 (t, $J = 6.8$ Hz, 6H, 26/27-H). ^{13}C NMR (101 MHz, CDCl_3): δ 170.73 (acetyl), 135.47, 131.90, 71.35 (C8), 56.31, 51.41, 42.79, 41.82, 39.90, 39.84, 33.05, 30.50, 27.47, 22.61, 21.31, 20.77, 19.93, 19.60, 17.90, 17.60, 13.17. ESI-HRMS: calculated for $\text{C}_{21}\text{H}_{36}\text{O}_2\text{Na}$ $[\text{M}+\text{Na}]^+$ 343.2613, found 343.2614.

2.2.7. (4S,7aR)-1-((2S,3R,4R,5R)-3,4-dihydroxy-5,6-dimethylheptan-2-yl)-7a-methyloctahydro-1H-inden-4-yl acetate (9)—To a solution of the compound **8** (3.3 g, 10.3 mmol) in acetone (40 mL) was added *N*-methylmorpholine *N*-oxide (6.0 g, 51.5 mmol) and 2.5% osmium tetroxide in *tert*-butanol (6.5 mL, 0.52 mmol) at room temperature with stirring. After 48 hours, water and ethyl acetate was added and the organic phase was separated, washed with brine and dried with Na_2SO_4 . Evaporation under vacuum gave the oily residue which was purified with flash chromatography on silica. Elution with hexane/ethyl acetate (10:1) gave pure compound **9** as colorless oil (2.44 g, 67%). ^1H NMR (400 MHz, CDCl_3): δ 5.14 (d, $J = 2.6$ Hz, 1H, 8-H), 3.71 (dd, $J = 2.4, 4.4$ Hz, 1H, 22-H), 3.59 (t, $J = 2.8$ Hz, 1H, 23-H), 2.39 (s, 1H), 2.04 (s, 4H), 1.94 – 1.79 (m, 2H), 1.70 (m, 5H), 1.55 – 1.09 (m, 9H), 1.00 (d, $J = 6.9$ Hz, 3H, 21-H), 0.96 (d, $J = 6.7$ Hz, 3H, 28-H), 0.92 – 0.84 (m, 9H, 18/26/27-H). ^{13}C NMR (101 MHz, CDCl_3): δ 170.80 (acetyl), 73.26 (C23), 71.19 (C8), 70.26 (C22), 53.02, 51.04, 44.11, 43.93, 42.45, 41.65, 40.00, 30.49, 29.89, 29.75, 26.91, 22.82, 21.44, 21.34, 18.94, 17.88, 13.91, 13.52, 12.83, 9.91. ESI-HRMS: calculated for $\text{C}_{21}\text{H}_{38}\text{O}_4\text{Na}$ $[\text{M}+\text{Na}]^+$ 377.2668, found 377.2668.

2.2.8. (4S,7aR)-7a-methyl-1-((S)-1-oxopropan-2-yl)octahydro-1H-inden-4-yl acetate (10)—To a suspension of compound **9** (2.2 g, 6.2 mmol) in THF- H_2O (4:1, 10 mL), sodium periodate (1.45 g, 6.82 mmol) was added at 0 °C in portions with stirring. After 1 hour, the reaction was quenched with ethylene glycol and the mixture was stirred at 0 °C for 10 minutes. Water was then added and the mixture was extracted with ethyl acetate for three times, washed with brine and dried with Na_2SO_4 . The combined extracts were evaporated to give crude oil, which was purified with flash chromatography on silica. Elution with hexane/ethyl acetate (5:1) gave pure compound **10** as transparent oil (1.03 g, 66%). ^1H NMR (400 MHz, CDCl_3): δ 9.57 (d, $J = 3.2$ Hz, 1H, CHO), 5.17 (d, $J = 2.0$ Hz, 1H, 8-H), 2.36 (m, 1H, 20-H), 2.05 (s, 3H), 1.97 (dt, $J = 12.3, 3.3$ Hz, 1H), 1.89 – 1.71 (m, 3H), 1.59 – 1.24 (m, 8H), 1.11 (d, $J = 6.8$ Hz, 3H, 21-H), 0.93 (s, 3H, 18-H). ^{13}C NMR (101 MHz, CDCl_3): δ 204.78 (CHO), 170.70 (acetyl), 70.89 (C8), 51.37, 50.74, 49.17, 42.47, 39.75, 30.47, 26.00, 22.95, 21.32, 17.83, 13.42, 13.35. ESI-HRMS: calculated for $\text{C}_{15}\text{H}_{23}\text{O}_2$ $[\text{M}-\text{H}_2\text{O}+\text{H}]^+$ 235.1698, found 235.1696.

2.2.9. (1S,4S,7aS)-1-acetyl-7a-methyloctahydro-1H-inden-4-yl acetate (12)—To a stirred solution of compound **10** (1.0 g, 3.9 mmol) in benzene (8 mL) was added morpholine (0.524 mL, 4.8 mmol) and *p*-toluenesulfonic acid (34mg, 0.195 mmol). The mixture was refluxed over a Dean-Stark water separator overnight. During the course of the reaction the theoretical amount of water (~70 mg) was separated. Upon completion as indicated by mass spectrum, the reaction mixture was cooled to room temperature and solvent was removed under vacuum to afford yellowish oil which was immediately used for next step.

To a stirred solution of the above crude mixture in acetone (20 mL) was added alumina (481 mg, 4.72 mmol) supported potassium permanganate (616 mg, 3.9 mmol) in portions. The reaction mixture was filtered after 2 hours and washed with ethyl acetate. Solvents were removed under vacuum to afford crude oily product which was purified with flash chromatography on silica. Elution with hexane/ethyl acetate (6:1) gave pure compound **12** as colorless oil (659 mg, 70% for two steps). ¹H NMR (400 MHz, CDCl₃): δ 5.16 (d, *J* = 2.8 Hz, 1H, 8-H), 2.48 (dd, *J* = 10.0, 8.0 Hz, 1H, 17-H), 2.20 – 1.99 (m, 8H), 1.92 – 1.82 (m, 1H), 1.70 – 1.39 (m, 8H), 0.81 (s, 3H, 18-H). ¹³C NMR (101 MHz, CDCl₃): δ 208.52 (C20), 170.26 (acetyl), 70.27 (C8), 63.54, 51.15, 43.06, 38.85, 31.26, 30.08, 22.54, 21.46, 20.98, 17.64, 14.51 (C18). ESI-HRMS: calculated for C₁₄H₂₂O₃Na [M+Na]⁺ 261.1467, found 261.1467.

2.2.10. (1S,4S,7aS)-1-((S)-2-hydroxy-6-methylheptan-2-yl)-7a-methyloctahydro-1H-inden-4-ol (**14**)

—To a stirred suspension of magnesium (181 mg, 7.5 mmol) in anhydrous THF (10 mL) was added 1-bromo-4-methyl-pentane (0.733 mL, 5.0 mmol) under argon at room temperature. The mixture was then refluxed for 2 hours and cooled down in ice bath to give Grignard reagent **13** which was used without further purification. Compound **12** (100 mg, 0.42 mmol) in 2 mL THF was subsequently added to the reaction mixture which was warmed to room temperature after 30 minutes and stirred overnight. Cooled saturated NH₄Cl solution was added to quench the reaction and the mixture was extracted with ethyl acetate for three times, washed with brine and dried with Na₂SO₄. The combined extracts were evaporated to give crude oil, which was purified with flash chromatography on silica. Elution with hexane/ethyl acetate (3:1) afforded pure compound **14** as colorless oil (94 mg, 80%). ¹H NMR (400 MHz, CDCl₃): δ 4.09 (d, *J* = 2.8 Hz, 1H, 8-H), 2.14 – 2.02 (m, 1H), 1.91 – 1.74 (m, 3H), 1.74 – 1.37 (m, 9H), 1.37 – 1.08 (m, 14H), 0.87 (dd, *J* = 6.7, 0.8 Hz, 6H, 26/27-H). ¹³C NMR (101 MHz, CDCl₃): δ 75.14 (C20), 69.41 (C8), 57.82, 52.55, 44.10, 42.52, 40.77, 39.56, 33.42, 27.92, 26.29, 22.71, 22.55, 22.21, 21.94, 21.30, 17.39, 15.44. ESI-HRMS: calculated for C₁₈H₃₁ [M-2H₂O+H]⁺ 247.2426, found 247.2430.

2.2.11. (1S,7aR)-1-((S)-2-hydroxy-6-methylheptan-2-yl)-7a-methylhexahydro-1H-inden-4(2H)-one (**15**)

—To a stirred solution of compound **14** (84 mg, 0.3 mmol) in dichloromethane (10 mL) was added pyridinium dichromate (225 mg, 0.6 mmol) at room temperature. The reaction mixture was stirred for 1 hour and filtered through celite. The celite was washed with dichloromethane and the combined solvent was removed under vacuum to give crude oily product which was purified with flash chromatography on silica. Elution with hexane/ethyl acetate (8:1) gave pure compound **15** as colorless oil (74 mg, 89%). ¹H NMR (400 MHz, CDCl₃): δ 2.43 (dd, *J* = 10.5, 6.9 Hz, 1H, 9-H), 2.33 – 2.13 (m, 3H, 9/12/14-H), 2.07 – 1.83 (m, 2H), 1.82 – 1.66 (m, 4H), 1.66 – 1.40 (m, 4H), 1.35 – 1.10 (m, 9H), 0.87 (d, *J* = 6.6 Hz, 6H, 26/27-H), 0.80 (s, 3H, 18-H). ¹³C NMR (101 MHz, CDCl₃): δ 211.99 (C8), 74.63 (C20), 62.10, 58.04, 49.84, 44.07, 40.80, 39.47, 39.26, 27.89, 26.53, 23.90, 22.67, 22.52, 21.95, 21.77, 18.73, 14.37. ESI-HRMS: calculated for C₁₈H₃₁O [M-H₂O+H]⁺ 263.2375, found 263.2377.

2.2.12. (1S,7aR)-1-((S)-2-(ethoxymethoxy)-6-methylheptan-2-yl)-7a-methylhexahydro-1H-inden-4(2H)-one (16)—To a stirred solution of compound **15** (67 mg, 0.24 mmol), *N,N*-diisopropylethylamine (83 μ L, 0.48 mmol) and 4-dimethylaminopyridine (12 mg, 0.096 mmol) in anhydrous dichloromethane (3 mL) was added chloromethyl ethyl ether (45 μ L, 0.48 mmol) dropwise at ice temperature. The resulting mixture was stirred for overnight at room temperature. Water was then added and the reaction mixture was extracted with dichloromethane, washed with brine and dried with Na₂SO₄. The combined extracts were evaporated under vacuum to give the oily residue which was separated with flash chromatography on silica. Elution with hexane/ethyl acetate (30:1) gave pure compound **16** as colorless oil (73 mg, 90%). ¹H NMR (400 MHz, CDCl₃): δ 4.81 – 4.64 (m, 2H, EOM), 3.69 – 3.45 (m, 2H, EOM), 2.41 (dd, *J* = 11.5, 7.0 Hz, 1H), 2.32 – 2.06 (m, 3H), 2.03 – 1.43 (m, 11H), 1.31 (s, 3H, 21-H), 1.26 – 1.09 (m, 7H), 0.86 (dd, *J* = 6.6, 0.9 Hz, 6H, 26/27-H), 0.76 (s, 3H, 18-H). ¹³C NMR (101 MHz, CDCl₃): δ 212.17 (C8), 88.76 (EOM), 79.75 (C20), 63.20 (EOM), 62.19, 57.31, 49.72, 40.83, 40.26, 39.70, 39.40, 27.87, 23.92, 23.21, 22.70, 22.57, 22.50, 21.70, 18.74, 15.14, 14.66. ESI-HRMS: calculated for C₂₁H₃₈O₃Na [M+Na]⁺ 361.2719, found 361.2719.

2.2.13. tert-butyl(((1S,Z)-3-((E)-2-((1S,7aS)-1-((S)-2-(ethoxymethoxy)-6-methylheptan-2-yl)-7a-methylhexahydro-1H-inden-4(2H)-ylidene)ethylidene)-4-methylenecyclohexyl)oxy)dimethylsilane (17)—To a stirred solution of compound **7** (51 mg, 0.15 mmol) in anhydrous THF (3 mL) was added phenyllithium (1.8 M in hexanes, 0.37 mmol) under argon at –78 °C. The mixture was stirred for another 30 minutes at this temperature and was added precooled compound **16** (167 mg, 0.37 mmol) in 100 μ L anhydrous THF. Reaction was kept stirring at this temperature for 3 hours and further 12 hours at room temperature. Water was then added and the reaction mixture was extracted with ethyl acetate, washed with brine and dried with Na₂SO₄. The combined extracts were evaporated under vacuum to give the oily residue which was purified with flash chromatography on silica. Elution with hexane/ethyl acetate (30:1) gave pure compound **17** as colorless oil (45 mg, 52%). ¹H NMR (400 MHz, CDCl₃): δ 6.16 (d, *J* = 11.2 Hz, 1H, 6-H), 6.01 (d, *J* = 11.2 Hz, 1H, 7-H), 5.02 (s, 1H, 19-H), 4.85 – 4.64 (m, 3H, 19-H and EOM), 3.81 (m, 1H, H-3), 3.71 – 3.50 (m, 2H, EOM), 2.82 (d, *J* = 12.4 Hz, 1H, 9-H), 2.44 (dd, *J* = 13.0, 4.2 Hz, 1H), 2.36 (dt, *J* = 13.5, 4.8 Hz, 1H), 2.28 – 2.17 (m, 1H), 2.16 – 1.93 (m, 3H), 1.93 – 1.79 (m, 2H), 1.77 – 1.44 (m, 13H), 1.39 – 1.08 (m, 12H), 0.87 (d, *J* = 7.7 Hz, 15H, 26/27-H and TBS), 0.67 (s, 3H, 18-H), 0.06 (d, *J* = 2.2 Hz, 6H, TBS); ¹³C NMR (101 MHz, CDCl₃): δ 145.34 (C10), 141.18 (C8), 136.36 (C5), 121.32 (C6), 118.21 (C7), 112.13 (C19), 88.80 (EOM), 80.42 (C20), 70.54 (C3), 63.14 (EOM), 57.38, 56.63, 46.84, 45.73, 40.98, 40.30, 39.79, 36.34, 32.73, 28.80, 27.94, 25.99, 25.96, 25.91, 25.88, 23.32, 22.97, 22.77, 22.55, 21.98, 21.91, 18.18, 15.19, 14.15, –4.59 (TBS), –4.63 (TBS). ESI-HRMS: calculated for C₃₆H₆₄O₃SiNa [M+Na]⁺ 595.4522, found 595.4509.

2.2.14. (1S,Z)-3-((E)-2-((1S,7aS)-1-((S)-2-hydroxy-6-methylheptan-2-yl)-7a-methylhexahydro-1H-inden-4(2H)-ylidene)ethylidene)-4-methylenecyclohexanol (1)—To a stirred solution of compound **17** (10 mg, 0.017 mmol) in MeOH-DCM (5:1, 3 mL) was added camphorsulfonic acid (4.3 mg, 0.020 mmol) at room temperature. The mixture was stirred for 3 hours and saturated NaHCO₃ was added.

The mixture was extracted with dichloromethane, washed with brine and dried with Na_2SO_4 . The combined extracts were evaporated under vacuo to give the oily residue which was purified with preparative TLC (hexane/ethyl acetate = 5:1) to afford pure compound **1** as colorless solid (2.6 mg, 37%). Mp: 91–93 °C (MeOH). ^1H NMR (400 MHz, CD_3OD): δ 6.22 (d, J = 11.2 Hz, 1H, 6-H), 6.02 (d, J = 11.2 Hz, 1H, 7-H), 5.08 – 5.00 (s, 1H, 19-H), 4.74 (d, J = 1.6 Hz, 1H, 19-H), 3.76 (m, 1H, 3-H), 2.85 (dd, J = 11.9, 4.0 Hz, 1H, 9-H), 2.58 – 2.48 (dd, 13.0, 4.2, 1H, 4-H), 2.40 (dt, J = 13.6, 4.9 Hz, 1H, 1-H), 2.25 – 1.91 (m, 5H), 1.81 – 1.62 (m, 5H), 1.61 – 1.43 (m, 6H), 1.43 – 1.09 (m, 11H), 0.89 (d, J = 6.6 Hz, 6H, 26/27-H), 0.69 (s, 3H, 18-H). ^{13}C NMR (101 MHz, CD_3OD): δ 147.00 (C10), 142.30 (C8), 137.34 (C5), 122.61 (C6), 119.37 (C7), 112.65 (C19), 75.99 (C20), 70.55 (C3), 59.89, 57.80, 47.02, 45.11, 42.25, 40.95, 36.60, 33.59, 29.84, 29.17, 26.15, 24.44, 23.16, 23.06, 23.02, 22.97, 14.14. ESI-HRMS: calculated for $\text{C}_{27}\text{H}_{44}\text{O}_2\text{Na}$ $[\text{M}+\text{Na}]^+$ 423.3239, found 423.3235.

2.3. VDRE luciferase reporter assay

Dulbecco's Modified Eagle Medium (DMEM) supplemented with glucose, *L*-glutamin, pyridoxine hydrochloride, together with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin antibiotic solution (Ab) (Sigma-Aldrich, St. Louis, MO) was used to culture immortalized human keratinocytes (HaCaT). Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% Ab. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 . Jurkat and HaCaT cells were transduced with lentiviral VDRE luciferase using Cignal Lenti VDRE Reporter (luc) Kit according to manufacturer's protocol (QIAGEN, Valencia, CA). After one week selection by puromycin (1 $\mu\text{g}/\text{mL}$), transduced HaCaT, Jurkat and Caco-2 cells were seeded in 96-well plate (1000 cells/well) using partial media (without FBS) for 24 h incubation. DMSO solutions of test compounds were added to cells, which were then incubated for another 24 h and measured for luciferase signal following the manufacturer's procedure of ONE-GloTM Luciferase Assay System (Promega, Madison, WI). The concentrations of DMSO were maintained consistently at 0.1% for compound treatment, and 0.1% DMSO were used as negative control. Concentration (100 nM) of 20S-(OH)D3 and 1,25(OH)₂D3 was used and tested in triplicates.

2.4. PCR

HaCaT cells were cultured as described above. The RNA from HaCaT keratinocytes treated with 20S-(OH)D3, 1,25(OH)₂D3 or DMSO, was isolated using the Absolutely RNA Miniprep Kit (Stratagen). Reverse transcription (100 ng RNA/reaction) was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Inc, Mannheim, Germany). Real-time PCR was performed using cDNA diluted 10-fold in sterile water and a SYBR Green PCR Master Mix. Reactions (in triplicate) were performed at 50 °C for 2 min, 95 °C for 10 min and then 40 cycles of 95 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec. Data were collected on a Roche Light Cycler 480. The amount of amplified product for each gene was compared to that of β -actin as a housekeeping gene using a comparative Ct method [25].

2.5. Anti-proliferation assay

To test anti-proliferative effect of 20S-(OH)D3 in comparison to 1,25(OH)₂D3 we measured [³H]-thymidine (Moravek Biochemicals Inc. Brea, CA) incorporation into the DNA of human SKEML-188 melanoma line as described previously[30]. Briefly, the cells were grown in 24-well plates using Ham's F10 media (Mediatech, Inc. Manassas, VA, USA) containing 5% charcoal treated FBS (Atlanta Biologicals, Lawrenceville, GA, USA) at 37°C in 5% CO₂ until 30% confluence. The cells were synchronized in serum free medium for 1 day and then grown in the media containing serum and 10⁻⁸ to 10⁻¹⁰ M of the compounds or vehicle for 72 hours. For the final 4 hours, the cells were incubated with [³H]-thymidine (final concentration of 0.5 µCi/mL). Afterwards media were replaced with 10% TCA in PBS (phosphate-buffered saline) and plates were washed twice with 1 mL PBS. The cells were lysed with 1 N NaOH/1% SDS, the extracts mixed with scintillation cocktail, and radioactivity was counted using Packard Matrix 9600 direct beta-counter (Packard, Meridan, CT, USA) as described previously [33].

3. Results

3.1. Chemistry

A retrosynthetic strategy was proposed as Scheme 1. The vitamin D3 scaffold could be established by Wittig-Horner coupling reaction between phosphine oxide **7** and C/D-ring fragment **16**. Due to steric effect of 18-methyl, stereoselective generation of 20S-hydroxy of **16** could be achieved using Grignard reagent **13** to attack **12**. In addition, introducing a relatively bulky protecting group on 8S-hydroxy was envisioned to assist the stereoselectivity of Grignard reaction. Conversion of VD2 to **7** and **12** could be carried out through oxidative cleavages.

The synthesis of phosphine oxide **7** and protected C/D-ring ketone **12** were performed following literature reports [35, 36]. To prepare **7** and **12** in large scale so as to facilitate future structure-activity relationship studies, we modified the synthesis which were outlined as Scheme 2 and Scheme 3. Initially, commercially available ergocalciferol was regioselectively dihydroxylated on the triene linkage to provide triol **2** with a yield of 65% when treating with potassium permanganate in ethanol at low temperature. Due to steric effect of 18-methyl, single 7*R*,8*R*-diastereoisomer was exclusively isolated over chromatography [35]. It is worth mentioning that when 3-OH was protected with TBS, dihydroxylation of the triene did not proceed. Subsequent treatment of vicinol **2** with lead tetraacetate in dichloromethane in the presence of sodium carbonate led to formation of corresponding C/D-ring ketone and A-ring allylic aldehyde. Although complete consumption of **2** was indicated on TLC, after workup procedures and purification using flash chromatography, A-ring allylic aldehyde ended up with poor yield (<20%) while C/D-ring ketone was obtained in 94% yield. We found that A-ring allylic aldehyde was unstable when exposed to air and moisture as clearly indicated by its spot on TLC and it should be used immediately. Thus, after quenching the oxidation reaction with ethylene glycol followed by simple workup procedure the crude oily mixture was directly used without purification to the following reduction step using excess equivalent of sodium bisaluminumhydride (65% in toluene) in benzene at 0 °C. Gratifyingly, these modified

procedures afforded pure A-ring allylic alcohol **3** and C/D-ring alcohol **4** with yield of 43% and 86% for two steps after chromatography, respectively. It is worth noting that after TLC indicated completion of the oxidation reaction, quenching the reaction by adding ethylene glycol was crucial to prevent further oxidation of the aldehyde to carboxylic acid. In addition, compared with lithium aluminum hydride (LAH) and diisobutylaluminum hydride (DIBAL-H in toluene or tetrahydrofuran), Red-Al (>65% in toluene) was more efficient and regioselective to reduce only aldehyde rather than conjugated diene to form **3**. Compound **3** was subsequently protected with TBS to generate **5**, which underwent selective removal of TBS on the allylic moiety to produce alcohol **6** in 71% yield for two steps when stirring with *tetra-n*-butylammonium fluoride (TBAF) in THF under low temperature (−5 °C to ice temperature). The final A-ring intermediate phosphine oxide **7** was prepared following literature procedures in three steps with 59% overall yield [36].

Acetate **8** was produced when secondary alcohol **4** was treated with acetic anhydride and pyridine. Compound **8** was subjected to Upjohn reaction stirring with osmium tetroxide (0.5% wt in *t*-butanol) and *N*-methylmorpholine *N*-oxide in acetone at ambient temperature for 48 hr to form the major diastereoisomer **9** due to strong steric effect of 21- and 24-methyl groups. While TLC indicated existence of the other diastereomer as minor product, **9** was isolated in 67% yield. Cleavages of vicinol **9** in the presence of sodium periodate and sodium carbonate in H₂O-THF (1:4) provided aldehyde **10** in 66% yield. Aldehyde **10** was then refluxed in a Dean-Stark apparatus stirring with morpholine and catalytic amount of *p*-toluenesulfonic acid in benzene to form crude enamine **11** as, after dried on vacuum, slightly yellowish oil. Due to its instability and sensitivity to moisture, the crude enamine mixture was used for next step immediately without further purification. While the removal of C-22 on the chiron could be accomplished when treating **11** with singlet oxygen [36], this photo-oxygenation procedure was rather tedious. By referring to an alternative strategy [37], the yellowish oil was dissolved in acetone and treated with alumina supported potassium permanganate that was made prior to use, to provide the other key intermediate **12** in 70% for two steps after chromatography.

As shown in Scheme 4, Grignard reagent **13** was readily accessible by refluxing commercially available 1-bromo-4-methyl-pentane with magnesium in anhydrous THF. After 2 hours, **13** was immediately subjected to Grignard reaction to form the diol **14** with 80% yield. Similar to our previous studies [33], only the 20*S*-epimer was isolated over chromatography due to the steric effect of both 18-methyl and 8-acetyl. This stereo-specificity is further supported by quantum mechanical calculations employing density functional theory (DFT) using Schrodinger Molecular Modeling Suite 2015. Figure 2 shows the thermodynamically most stable and the preferred orientation of the acetyl group for the intermediate **12**. This conformational preference dictated the strongly favored back-side attack by the Grignard reagent **13** to form **14** with 20*S*-OH configuration. The attack from the front side to form 20*R*-OH configuration is sterically prohibited, consistent with what we found previously [33, 38]. Oxidation of diol **14** using Cornforth reagent pyridinium dichloromate (PDC) gave 20*S*-hydroxy Grundmann's ketone **15** in 89% yield. Compound **15** was subsequently introduced protecting group EOM on its tertiary alcohol to form ketone **16**, the other key intermediate for the final coupling step. With both C/D-ring fragment **16**

and phosphine oxide **7** in hand, construction of the 20S-(OH)D3 framework was successfully accomplished by employing Wittig-Horner reaction in the presence of phenyllithium (0.18 M, diluted from commercial source) in THF to form **17** in 52% yield. Simultaneously removal of EOM and TBS was achieved using camphorsulfonic acid (CSA) to produce the target compound 20S-(OH)D3 in 37% yield.

3.2. Biological assays

3.2.1. 20S-(OH)D3 activates VDR—To confirm that the synthetic 20S-(OH)D3 can activate VDR, two cell lines (Jurkat and HaCaT) were transduced by VDRE luciferase vector to establish corresponding reporter assays. We compared the VDR activation activity of 20S-(OH)D3 with that of 1,25(OH)₂D3 as shown in Figure 3. At a concentration of 100 nM, both 20S-(OH)D3 and 1,25(OH)₂D3 can activate VDR as compared with control in both cell lines, however, 1,25(OH)₂D3 showed much stronger activity than 20S-(OH)D3 suggesting deficiency in coupling of 20S-(OH)D3 with activation of genes having VDRE consensus in their promoter. These results are consistent with results obtained using enzymatically generated 20S-(OH)D3 [25] indicating that 20S-(OH)D3 acts as a biased agonist on the VDR [23].

3.2.2. 20S-(OH)D3 stimulates the mRNA levels of VDR and CYP24A1—Since the CYP24A1 gene is an important target of 1,25(OH)₂D3 activation, we compared the action of 20S-(OH)D3 with that of 1,25(OH)₂D3 on the expression of VDR itself and CYP24A1 genes in HaCaT cells at different time points as shown in Figure 3. After 6 hrs of treatment with 100 nM of the compound, cells exposed to 1,25(OH)₂D3 showed 350 fold increased expression of CYP24A1 and only 2- to 3-fold after treatment with 20S-(OH)D3, which is consistent with previously reported data [23]. Interestingly, 20S-(OH)D3 is much stronger inducer of VDR gene expression than 1,25(OH)₂D3 (Fig. 3B).

3.2.3. Anti-proliferation activity of 20S-(OH)D3—As shown in Figure 4, 20S-(OH)D3 showed potent anti-proliferative and dose-dependent effect on SKMEL-188 melanoma cells comparable to classical active form of vitamin D3, 1,25(OH)₂D3. 20S(OH)D3 gave an IC₅₀ of 2.09×10^{-10} M while the IC₅₀ of 1,25(OH)₂D3 was 2.78×10^{-9} M. This result is consistent with previous studies showing that 20S-(OH)D3 shows equal or higher potency for inhibition of melanoma cell proliferation in comparison to 1,25(OH)₂D3 [27, 39].

4. Discussion

20S-(OH)D3, a noncalcemic vitamin D3 analog, was enzymatically converted from vitamin D3 by P450_{scc} and was first chemically synthesized via UVB irradiation [33]. However, this photochemical method also produces structurally similar and physicochemically active by-products that present a significant challenge for purification and further SAR studies for the promising 20S-hydroxyl scaffold. Therefore, we seek to develop an alternative synthetic method. The total synthetic strategy described in this report involves Wittig-Horner coupling and Grignard reaction and is successful in generating 20S-(OH)D3 in 0.4% overall yield in 16 steps. A distinct advantage of this method over our previously reported method is that after we generate the two key intermediates phosphine oxide **7** and C/D-ring ketone **12** on

gram scale from a common starting material vitamin D₂, we can efficiently make side chain derivatives for in-depth SAR studies of this 20S-hydroxyl scaffold.

As the native ligand of VDR, 1,25(OH)₂D₃ activated VDR (Figure 5) and initiated the rapid transcriptional activity inside the nucleus. This included highly enhanced expression of CYP24A1 (~ 350-fold) after 6 h treatment. In contrast, 20S-(OH)D₃ showed weak stimulation of CYP24A1 gene expression consistent with our previous data identifying 20S-(OH)D₃ as the biased agonist for the VDR [23]. Enhanced expression of VDR by treatment with 20S-(OH)D₃ with higher potency than that of 1,25(OH)₂D₃ further emphasized certain differences with this classical agonist, and suggested amplification of VDR mediated effects, a subject warranting further studies.

In conclusion, we have established an efficient total synthesis of biologically active 20S-(OH)D₃, which involved divergently generation of key intermediates **7** and **12** from a common starting material VD₂ via modified methods, stereoselective formation of the 20S-hydroxyl scaffold through Grignard reaction and simultaneously removal of EOM and TBS using CSA. Results from various biological assays demonstrated the similarity of 20S-(OH)D₃ produced through this total synthesis method with the authentic compound generated enzymatically. Further modifications on the side chain to generate 20S-(OH)D₃ analogs for SAR study will be carried out via nucleophilic attack on intermediate ketone **12** before it will be coupled to the other key intermediate **7**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

D3	Vitamin D3
20S-(OH)D3	20S-hydroxyvitamin D3
1,25(OH)₂D3	1α,25-dihydroxyvitamin D3
EOM	Ethoxymethyl
TBS	<i>t</i> -butyldimethylsilyl

Highlights

- Key intermediates **7** and **12** were generated in parallel via modified synthetic ways
- Simultaneous removal of EOM and TBS was completed using camphorsulfonic acid
- 20*S*-hydroxyvitamin D3 was synthesized in 16 steps with an overall yield of 0.4%
- Synthetic 20*S*-(OH)D3 activated VDR and initiated expression of downstream genes
- 20*S*-(OH)D3 was potent in inhibition of melanoma cells proliferation

Fig. 1.
Marketed vitamin D analogs and noncalcemic 20S-(OH)D3

Fig. 2. Preferred conformation of the intermediate (compound **12**) calculated with density function theory with 6-31G** baseset. "Front" side attack by the bulky Grignard agent **13** to form 20*R* isomer of **14** is prohibited due to the steric hindrance from the 18-methyl and other moieties in compound **12**, while the "back" side attack to form 20*S* isomer is strongly favored.

Fig. 3. 20S-(OH)D3 stimulates mRNA levels of VDR and CYP24A1 at 6 h (A) and 24 h (B). HaCaT cells were treated for 6 h with 100 nM of 20S-(OH)D3, 1,25(OH)₂D3 or DMSO only (solvent) as a control. The mRNA was isolated and qPCR performed using specific primers for VDR and CYP24A1 genes. Data are presented as mean ± SE (n = 3, * p < 0.05, *** p < 0.005, **** p < 0.0001).

Fig. 4. Anti-proliferative effects of 20S-(OH)D3 and 1,25(OH)₂D3 on human SKEML-188 melanoma cells. Cells were treated with 20S-(OH)D3, 1,25(OH)₂D3 or DMSO (solvent) as a control to assess their inhibitory effects on DNA synthesis. Data are presented as mean $\pm$ SE (n = 3, * p < 0.05, ** p < 0.01, *** p < 0.005).

Fig. 5. 20S-(OH)D3 activates luciferase activity in VDRE-LUC transduced Jurkat and HaCaT cells. Cells were treated for 24 h with 100 nM 20S-(OH)D3, 1,25(OH)₂D3 or DMSO as a control. Data are presented as mean $\pm$ SE (n = 3, * p < 0.05, ** p < 0.01).

Scheme 1.
Retrosynthetic analysis of 20S-(OH)D3

Scheme 2.

Synthesis of intermediate **7** Reagents: (a): KMnO₄, EtOH-H₂O; 65%; (b): i. Pb(OAc)₄, Na₂CO₃, DCM; ii. Red-Al, benzene; 43% for **3** and 86% for **4** in two steps; (c): TBSCl, Imidazole, DCM; 88%; (d): TBAF, THF; 78%; (e): i. *n*-BuLi, *p*-TsCl, THF; ii. *n*-BuLi, Ph₂PH, THF; iii. H₂O₂, H₂O, DCM; 59% in three steps.

Scheme 3.

Synthesis of intermediate **12** Reagents: (a): Ac_2O , Pyr, DMAP, DCM; 92%; (b): OsO_4 , NMO, Acetone/*t*-BuOH; 67%; (c): NaIO_4 , H_2O -THF; 66% (d): Morpholine, *p*-TsOH, PhH, reflux; (e): $\text{KMnO}_4/\text{Al}_2\text{O}_3$, Acetone; 70% in two steps.

Scheme 4.

Synthesis of 20S-(OH)D3 Reagents: (a): Mg, THF; (b): THF; 80% (c): PDC, DCM; 89%; (d): EOMCl, DMAP, TEA, DCM; 90%; (e): **7**, PhLi, THF; 53%; (f): CSA, DCM-MeOH; 37%.